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## GLYCEROL METABOLISM IN THE PLAGUE MICROBE

Following is the translation of an article by I. V. Domaradskiy, L. V. Linnikova, and Ye. P. Golubinskiy, Scientific-Research Antiplague Institute, Rostov/Don, published in the Russian-language periodical Voprosy Meditsinskoy Khimii (Problems of Medical Chemistry) 14(2), 1968, pages 185--190. It was submitted on 26 Aug 1966.]

A different relation to glycerol is one of the main features which make it possible to distinguish variants of the plague microbe from each other [1, 2]. The question of why some of these ferment glycerol and others do not remains unanswered up til now. We decided to study the mechanism of fermentation of glycerol in cultures of glycerolpositive strains of the plague microbe, and by means of a comparison of strains which decompose and do not decompose glycerol to clear up the absence of which enzyme (enzymes) conditions the inability of the microbes to ferment glycerol. \* Since both types of strains ferment glucose easily in the same manner in the set up of Embden-Meyerhof [3], we turned our main attention to the study of the initial phases of conversion of glycerol, up to the stage of phosphotriose. In this communication certain preliminary results of our investigations are presented.

\* Subsequently we designate glycerolpositive strains as  $GL^+$ , and glycerolnegative -  $GL^-$ .

## Methods

The tests were set up with vaccine strains EV ( $GL^-$ ) and No 17 ( $GL^+$ ). In addition to this in one series of tests we used the prototrophic mutants of these strains, vaccine strain No 1 ( $GL^-$ ) and virulent strain No 1210 ( $GL^+$ ) \*\*. For investigating the metabolism of glycerol and its possible metabolites the bacteria were incubated on a thick Hottinger medium or casein hydrolyzate (pH 7.2), with glycerol as a rule, for 2 days at 28°. The suspensions of cells were washed three times with a physiological solution of sodium chloride. The concentration of cells was established by the optical standard of the Control Institute imeni L. A. Tarasevich. Absorption of oxygen was measured by the "direct" method in a Warburg apparatus at 37°. Samples usually contained 1.2 ml of buffer (phosphate M/15, veronal M/10, borate M/15), of the corresponding pH, 0.2 ml of substrate (most often 18 micromoles), and 0.5 ml of cell suspension ( $5 \cdot 10^{10}$ ). In the control samples in

place of the substrate water was added. For studying the metabolism of glycerol under anaerobic conditions tests were set up at 37° in test tubes under a layer of sterile liquid petrolatum (composition of the sample remained the same). Glycerol was determined by the method described by Weish [4], pyruvate - by the method of Frideman and Khaugen, lactate - by the method of Barker and Samerson, phosphorus - by the method of Fisk-Subbarow. The utilization of glycerol and its possible metabolites was judged by the capacity of the plague microbe to grow on synthetic media, in which these substances were main (in the case of auxotrophic strains) or unique (in the case of prototrophic mutants) sources of carbon. During work with the prototrophic mutants we used a medium which was prepared by means of the addition to the saline base of the synthetic medium [5] of 0.05% ammonium sulfate, 0.05% of one of the investigated preparations, and 1.5% of washed agar. In experiments with auxotrophic strains the synthetic medium, in addition to the stated ingredients, contained 0.1 mg% of phenylalanine, 0.25 mg% of cysteine, 0.1 mg% of methionine, and 0.1 mg% of threonine (in the absence of additional sources of carbon the stated amino acids in the cited concentrations did not support the growth of the plague microbe). Inoculations of washed cultures of plague microbe on synthetic media were made with a calculation of 10<sup>6</sup> microbial cells per dish. Results of the experiments were considered after incubation of seedings at 28° for 5 days.

\*\* Subsequently the initial strains EV and No 17, and also No 1 and 1210 are designated as auxotrophic.

#### Results of the Investigations

As preliminary experiments showed, strain GL<sup>+</sup> of the plague microbe decomposes glycerol with the absorption of oxygen; in contrast to strain GL<sup>+</sup> strain GL<sup>-</sup> does not oxidize glycerol. Decomposition of glycerol by strain GL<sup>+</sup> is primarily an aerobic process, under anaerobic conditions the decrease of glycerol is 86% less. In the latter case it still has not been cleared up as to what is the acceptor of hydrogen.

The capacity of strain GL<sup>+</sup> to oxidize glycerol is strengthened considerably if it is incubated on a medium with glycerol (see drawing). Therefore we set up all the subsequent experiments with adapted cultures. The addition of glucose to the medium is not noticeably reflected in the ability of the plague microbe to oxidize glycerol. This fact is interesting due to the fact that on certain other properties of the plague microbe glucose exerts a negative effect [6].

The capacity of the plague microbe to decompose glycerol is preserved after washing and aeration or storage of cells at 5°, but disappears under the influence of toluene. The effect of other

enzyme poisons on the catabolism of glycerol is shown in Table 1. As can be seen, a significant influence on the metabolism of glycerol is exerted only by bromo- and iodoacetic acid: with concentrations of them equal to  $10^{-3}M$ , absorption of oxygen and consumption of glycerol are completely stopped.

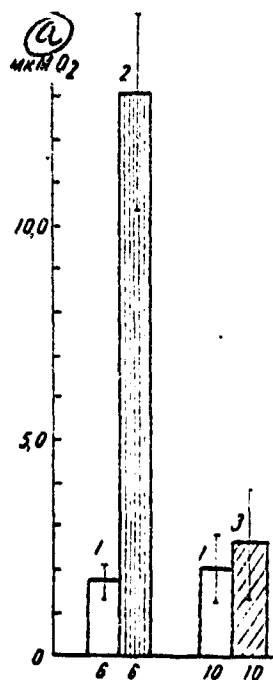
In a borate buffer the optimum of the oxidation reaction of glycerol lies at pH 7.8. In a veronal buffer in the range of pH 7.0--9.0 it was not possible to establish an expressed optimum, but at pH 6.0 the absorption of oxygen and decrease of glycerol are reduced. At pH 7.2 the intensity of conversion of glycerol in a borate, and especially in a veronal, buffer is comparable with the rate of oxidation in a phosphate buffer (Table 2).

Judging by our data, for 1 mole of decomposing glycerol an average of around 1 mole of oxygen is consumed. Stoichiometrically this corresponds to the conversion of glycerol into pyruvic acid\*\*\*. However, we never observed the conversion of glycerol into pyruvate even in a veronal buffer, in which pyruvate usually accumulates most of all (see Tables 1 and 2). Apparently the process does not stop at the stage of formation of pyruvate; it proceeds further, but already without a noticeable expenditure of oxygen. Here, along with other, still unidentified, products lactic acid is formed, but the quantity of it does not exceed 10% (2 micromoles) of the amount of oxidized glycerol.

\*\*\* It is necessary to take into consideration that due to shortcomings in the design of the manometric vessels (one stationary side retort) the time of determination of decrease of glycerol in our experiments always exceeded the time of calculation of oxygen consumption. This apparently explains the quantitative difference in the decrease of glycerol and the consumption of oxygen.

As is known, the decomposition of glycerol begins with its phosphorylation or dehydrogenation. In order to come close to an answer to the question as to what starts the decomposition of glycerol in the plague microbe, we studied the relation of  $GL^+$  and  $GL^-$  strains of the plague microbe to glycerophosphate, dihydroxyacetone, and glyceraldehyde. The results of our experiments are reduced to the following. The capacity for the oxidation of d,l-alpha-glycerophosphate is possessed only by the  $GL^+$  strain, and then under the condition that the experiments are conducted in a veronal buffer, but even in this case the rate of oxidation of glycerophosphate is less than the rate of oxidation of glycerol (Tables 3 and 4). The oxidation of alpha-glycerophosphate is accompanied by the liberation of inorganic phosphate in quantities which are equivalent to the amount of absorbed oxygen, and by the formation of traces of pyruvic acid. It is interesting to note that the beta-isomer of glycerophosphate, which is not an intermediate product from the decomposition of glycerol, is also oxidized by the  $GL^+$  strain of the plague microbe. In spite of the fact that the

GL<sup>-</sup> strain does not oxidize both isomers of glycerophosphate, small amounts of inorganic phosphate and glycerol are detected in the medium. Apparently in this case decomposition is realized under the influence of phosphatase [7, 8]. The GL<sup>+</sup> and GL<sup>-</sup> strains of the plague microbe either do not oxidize dihydroxyacetone at all or oxidize it very weakly. In contrast to dihydroxyacetone, d,l-glyceraldehyde is oxidized by the GL<sup>+</sup> strain (but not the GL<sup>-</sup>). Here the maximum rate of oxidation is noted in a phosphate buffer (see Table 4). For the purpose of determining which substrates are used by the plague microbe as unique sources of carbon in the medium, we used the prototrophic mutants of the plague microbe [9]. As the experiments showed (Table 5), on minimum media the GL<sup>+</sup> mutants grow in the presence of glycerol, alpha- and beta-isomers of glycerophosphate, and 3-phosphoglyceric acid; glycerolnegative mutants grow only in the presence of the last substrate. Behaving in the same manner as the GL<sup>+</sup> and GL<sup>-</sup> prototrophic strains are their corresponding auxotrophic strains of the plague microbe. It is important to stress that under the given conditions dihydroxyacetone and glyceraldehyde are not sources of carbon for the plague microbe.



Influence of glycerol and glucose in the incubation medium on the capacity of the plague microbe to oxidize glycerol. Voronin buffer pH 7.2; incubation 25 minutes; reliable interval - for probability 0.99.

- 1 - absorption of oxygen by culture from a medium without an additive;
  - 2 - absorption of oxygen by culture from a medium with glycerol;
  - 3 - absorption of oxygen by culture from a medium with glucose.
- Figures under the columns - number of tests. (a) - micromoles.

Table 1

Influence of enzyme poisons on the metabolism of glycerol (strain No 17, pH 7.2, incubation 25 minutes; in micromoles per sample) 1

Проба (a)	Число опытов (b)	Концентрация яда (в молях) (c)	Буфер (d)	Поглощено кислорода (e)	Использовано глицерина (f)	Обнаружено пировиноградной кислоты (g)
× Без яда	5	—	Фосфатный (h)	10,7 ± 0,69	13,6 ± 0,87	0,5 ± 0,14
○ С ДНФ	5	10 <sup>-4</sup>	»	12,8 ± 0,69	15,8 ± 0,81	0,3 ± 0,11 (i)
× Без яда	6	—	Вероналовый (j)	12,3 ± 0,39	15,9 ± 0,47	7,4 ± 0,51
○ С ДНФ	6	10 <sup>-4</sup>	»	10,3 ± 0,72	15,2 ± 0,53	8,2 ± 0,52
× Без яда	4	—	»	11,2 ± 0,63	14,7 ± 1,31 (k)	—
● С ФН	4	5 × 10 <sup>-4</sup>	»	9,8 ± 0,64	13,6 ± 0,91 (l)	—
× Без яда	3	—	Фосфатный (h)	12,0 ± 1,21	16,4 ± 0,32	—
Δ С МНУК	1	10 <sup>-3</sup>	»	0	0	—
Δ С МНУК	1	10 <sup>-4</sup>	»	7,4	—	—
Δ С МНУК	1	2 · 10 <sup>-4</sup>	»	3,4	4,2	—
× Без яда	7	—	Вероналовый	11,5 ± 0,54	16,1 ± 0,21	—
Δ С МНУК	3	10 <sup>-3</sup>	» (l)	0,4 ± 0,21	0	—
Δ С МНУК	3	10 <sup>-4</sup>	»	7,7 ± 0,47	7,7 ± 0,46	—
Δ С МНУК	1	2 · 10 <sup>-4</sup>	»	1,8	2,2	—
× Без яда	4	—	»	11,2 ± 0,42	16,2 ± 0,45 (m)	2,8 ± 1,35
□ С МФАА	4	10 <sup>-3</sup>	»	11,7 ± 0,67	17,1 (n)	3,1 ± 1,48
× Без яда	4	—	»	11,4 ± 0,86	11,4 ± 0,92	4,9 ± 1,60
■ С МВУК	3	10 <sup>-3</sup>	»	0	0,9 ± 0,33	0
■ С МВУК	1	10 <sup>-4</sup>	»	8,7	9,0	3,4

1 In this and subsequent tables the figures represent the average and the error of averages ( $M \pm m$ ).

2 [Acronyms explained in the legend]

3 Average of 3 tests.

4 Average of 2 tests.

5 One test.

Legend: (a) Sample: x - Without poison; o - with DNP /p-dinitrophenol/;

● - With NaF /sodium fluoride/; Δ - With IOA /iodoacetic acid/;

□ - With MFAA /monofluoroacetamide/; ■ - With BAA /bromoacetic acid/;

(b) Number of tests; (c) Concentration of poison (in moles); (d)

Buffer; (e) Absorbed oxygen; (f) Utilized glycerol; (g) Pyruvic

acid detected; (h) Phosphate; (i) Veronal.

Table 2

Metabolism of glycerol in various buffers (pH 7.2, strain No 17, incubation 40 min; in micromoles per sample)

Буфер (a)	Число опытов (b)	Поглощено кислорода (c)	Использовано глицерина (d)	Обнаружено пировиноградной кислоты (e)
(f) Фосфатный	6	12,8 ± 0,81	11,9 ± 1,31	0,7 ± 0,36
(g) Вероналовый	5	11,9 ± 1,05	15,2 ± 1,22	8,4 ± 0,95
(h) Боратный	4	9,3 ± 0,07	7,1 ± 1,00	1,0 ± 0,32

Key: (a) Buffer; (b) No of tests; (c) Oxygen absorbed; (d) Glycerol used; (e) Pyruvic acid detected; (f) Phosphate; (g) Veronal; (h) Borate.

## Discussion of Results

Our findings testify to the presence in GL<sup>+</sup> strains of a number of enzymes which participate in the decomposition of glycerol and which are not formed by GL<sup>-</sup> strains. Consequently the difference between the GL<sup>+</sup> and GL<sup>-</sup> strains amounts to the inability of GL<sup>-</sup> strains to form all these enzymes. The latter is probably a genotypic characteristic. As regards the question of the paths of conversion of glycerol in GL<sup>+</sup> strains, then it still remains open. In the light of the data presented, the previously expressed hypothesis that glycerophosphate does not lie on the path of breakdown of glycerol [10] has received new confirmation. However, not included within the framework of this hypothesis are facts testifying to the absence in the plague microbe of the capacity to oxidize dihydroxyacetone and to use it as a source of carbon. Nevertheless, the first product in the conversion of glycerol may not be dihydroxyacetone, but glyceraldehyde.

Table 3

Comparative data on the metabolism of glycerol and d,l-alpha-glycerophosphate (veronal buffer, pH 7.2, incubation 25 min, strain No 17, in micromoles per sample)

(a) Субстрат окисления	(b) Число опытов	(c) Поглощено кислорода	(d) Обнаружено пирувено-градной кислоты	(e) Обнаружено фосфата	(f) Использовано глицерина
(g) Глицерин	5	11,7±0,79	9,5±1,11	—	15,0±0,91
(h) Глицерофосфат	5	7,1±0,55	1,3±0,39	6,5±0,03	—

Key: (a) Oxidation substrate; (b) Number of tests; (c) Oxygen absorbed; (d) Pyruvic acid detected; (e) Phosphate detected; (f) Glycerol used; (g) Glycerol; (h) Glycerophosphate.

For a final solution of the question concerning the paths of glycerol breakdown it is necessary, first of all, to obtain preparations of the corresponding enzymes, and, secondly, to attempt to isolate a mutant which is devoid of phosphatase activity (relative to alpha-glycerophosphate). This will make it possible to exclude the possibility of oxidation of glycerophosphate by an intermediate route, i.e., through glycerol. Finally it is necessary to experimentally verify the hypothesis of Boyce and associates [11] concerning the metabolism of glycerol in the plague microbe through the stage of formation of hexose, and to clear up in what manner the GL<sup>-</sup> strain of the plague microbe uses glycerin for the synthesis of amino acids, ribose, and deoxyribose.

Table 4

Absorption of oxygen in the presence of glycerol and its possible metabolites (pH 7.2, incubation 25 min; in micromoles per sample)

Субстрат окисления (a)	Число опытов (b)	Штамм № 17 (c)		Штамм № 17 (d)	
		фосфатный буфер (e)	верональный буфер (f)	фосфатный буфер (g)	верональный буфер (h)
g Глицерин	11	13.9 ± 0.83	14.1 ± 0.81	0	0
h d, l-α-глицерофосфат	4	1.8 ± 0.25	7.0 ± 0.56	0.2	0
i Дигидроацетон	9	0.8 ± 0.16	1.5 ± 0.29	0.2	0
j d, l-глицеральдегид	5	13.4 ± 0.75	2.0 ± 0.42	0	0

Key: (a) Oxidation substrate; (b) number of tests; (c) Strain No 17; (d) strain EV; (e) phosphate buffer; (f) Veronal buffer; (g) Glycerol; (h) d,l-α-glycerophosphate; (i) dihydroxyacetone; (j) d,l-glyceraldehyde.

Table 5

Growth of plague microbe on dense synthetic media

Штамм чумного микроба (a)	Число опытов (b)	Рост штаммов чумного микроба в присутствии (c)					
		глицерин (d)	глицеральдегид (e)	глицерофосфат (f)	диоксинацетон (g)	3-фосфоглицерическая кислота (h)	без источников углерода (контроль) (i)
EV прототроф GL <sup>-</sup>	5	—	—	—	—	+	—
№ 17 прототроф GL <sup>+</sup>	5	+	—	+	—	+	—
EV ауксотроф GL <sup>-</sup>	1	—	—	—	—	+	—
№ 17 ауксотроф GL <sup>+</sup>	1	+	—	+	—	+	—
№ 1 ауксотроф GL <sup>-</sup>	2	—	—	—	—	+	—
№ 1210 ауксотроф GL <sup>+</sup>	2	+	—	+	—	+	—

Legend: + growth; — absence of growth.

Key: (a) Strain of plague microbe; (b) Number of tests; (c) Growth of plague microbe strains in the presence of; (d) glycerol; (e) glyceraldehyde; (f) glycerophosphate; (g) dihydroxyacetone; (h) 3-phosphoglyceric acid; (i) without sources of carbon (control); (j) EV prototroph GL<sup>-</sup>; (k) No 17 prototroph GL<sup>+</sup>; (l) EV auxotroph GL<sup>-</sup>; (m) No 17 auxotroph GL<sup>+</sup>; (n) No 1 auxotroph GL<sup>-</sup>; (o) No 1210 auxotroph GL<sup>+</sup>.

### Conclusions

1. The GL<sup>+</sup> strain of the plague microbe oxidizes glycerol, glyceraldehyde, and glycerophosphate, but practically does not oxidize dihydroxyacetone. The GL<sup>-</sup> strain does not oxidize those substrates at all.
2. As unique sources of carbon the GL<sup>+</sup> strain uses glycerol, glycerophosphate, and 3-phosphoglyceric acid. The GL<sup>-</sup> strain uses only the last substrate.
3. The capacity to oxidize glycerol is strengthened considerably during incubation of the GL<sup>+</sup> strain on a medium with glycerol.
4. Oxidation of glycerol is accompanied by the formation of pyruvic acid and lactic acid.
5. Oxidation of glycerol is inhibited by bromo- and iodoacetic acid; such an effect is not exhibited by dinitrophenol, fluoroacetamide, and sodium fluoride.
6. The influence of the nature of the buffer on the breakdown of glycerol is manifested only in the fact that in a veronal buffer there is a greater accumulation of pyruvic acid than in the others. In a veronal buffer the greatest rate of oxidation lies within the range of pH 7.0--9.0.

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